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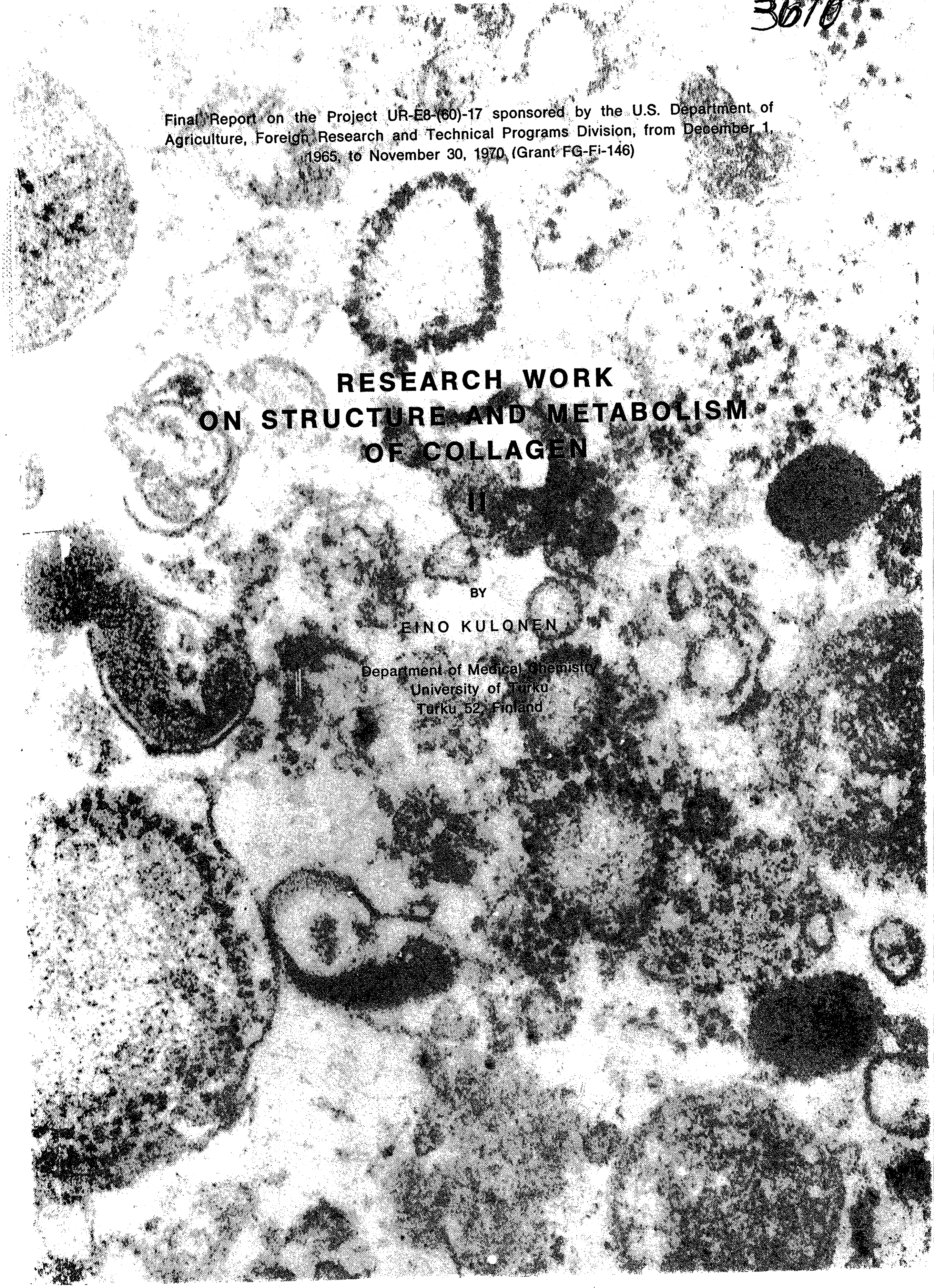

Final Report on the Project UR-E8-(60)-17 sponsored by the U.S. Department of Agriculture, Foreign Research and Technical Programs Division, from December 1, 1965, to November 30, 1970 (Grant FG-Fi-146)

RESEARCH WORK ON STRUCTURE AND METABOLISM OF COLLAGEN

II

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CONTENTS

GENERAL SUMMARY

Purpose

This work was made to increase our knowledge on the connective tissue, especially on the collagenous fibres, with the practical applications in mind. Because the aim was not specified in more detail, the scope of the work was rather broad with long-range expectations.

Results

1. *Some essential features of the collagen structure have been revealed by the comparative work on materials from various species and tissues. A scheme of the relationships of different collagens is suggested. This information can be utilized for the eventual modifications of collagenous preparations (fibres, sheets, polymerizable macromolecules) on stability (thermal or metabolic) or on physical properties (tensile strength, solubility).*

2. *Different techniques for the degradation of the connective tissue have been worked out with the modern methods of protein chemistry. They should be useful in further work on collagenous tissues and on gelatins. A method has been developed for the independent estimation of a frequent dipeptide in collagen, prolyl-hydroxyproline.*

3. *Attention has been paid to the formation of collagenous polymers, which involves both non-enzymatic and enzymatic phases. A part of "cold-insoluble" collagen dissolves at +40° already which indicates linkages of varying strength in the supermolecular structure. The aggregation is influenced by certain additions in very low concentrations (e.g., 1 mole ethanol among 1000 moles of water). The active substances include alcohols, amino acids, divalent cations and nucleoside triphosphates. The formation of insoluble collagenous tissue in vitro is sensitive to aminoacetonitrile and semicarbazide, presumably at the deamination of lysine. Calcium in physiological concentrations and other cations as also non-collagenous proteins are important in this process. The formation of insoluble collagen occurs most efficiently in young animals. Knowledge on these phenomena is important for the eventual design of collagenous materials to polymerize in situ. Such synthetic products exist already for medical purposes*

4. *The work on the synthesis of collagen has an economic interest from the point of animal husbandry and of the veterinary medicine. The turn-*

over of collagen in a living animal can be influenced by manipulations of the diet, of the supply of oxygen, of the hormonal balance or by administering drugs. Practical applications might emerge in the application of connective tissue components to the promotion of wound healing.

5. *In the appendix relevant metabolic work with collagen-producing tissues is presented to indicate biological aspects on the regulation of fibroblast activities.*

Future prospects

Considering the large investments made by the chemical industry on the polymer chemistry, I feel that this grant has produced a satisfactory return. The ways which Nature applies in handling collagen have to be understood completely before its full economical exploitation. The goals of the work on the utilization of connective tissue should be defined better.

At present the synthetic materials are cheaper almost for any purpose. For example, for closing the wounds entirely synthetic alkylated cyanoacrylates are gaining ground. When demand appears for a material which should have the properties of collagen (variable thermal stability, tissue compatibility, polarity, capacity to aggregation, mechanical strength), a large specific research investment is justified. Meanwhile the work motivated by the present ignorance should continue.

INTRODUCTION

Evolution of collagen has occupied a rather conspicuous role in our studies but also produced the most original results. This was not so planned but our interest was directed by an incidental observation. Some essential features of collagen, also on the supermolecular level, have been disclosed. The "atypical" collagens of cartilage and basement membrane are included to a comprehensive scheme.

The chapter on the structure of collagen refers to procedures of degradation which are applicable to structural studies of the insoluble connective tissue components. Observations on the influence of age and associated non-collagenous components are enclosed.

Formation of collagen is initiated by intracellular synthesis at the fibroblast level and continued by the aggregation and maturation. This kind of study seems to be most rewarding in the future, also from the practical point of view. In the production of cattle, the cost of collagen in the carcass should be assessed with reference to its turnover which is much lower than, *e.g.*, of muscle proteins. Would it be possible to decrease the turnover of collagen in skin and bone, less food should be required for the formation and maintenance of the skeleton and body wall.

Regulation of the activities of the fibroblasts does not strictly belong to the project sponsored by U.S. Department of Agriculture and is therefore presented in an Appendix. Preliminary findings are included in this report to stress the significance of the biological aspects and to suggest a direction of future research.

Fibrous collagen

Our present ideas on the evolution of collagen are summarized in two papers (*6, 7*). Fig. 1 shows the suggested divergent evolution with certain approximations and speculations. The original results are presented in the thesis of Dr. J. Pikkarainen (*8*), in a report on invertebrates (*9*), in a short manuscript of carbohydrates of collagen (*60*) and in two congress reports (*10, 11*). A. preliminary attempt with CNBr-degradation of various collagens (*12*) shows that further study on the primary structures would be rewarding.

The main force of evolution has been the trend to more stable forms, both thermally and metabolically. This has been possible through the proportional increase of the imino acids, which replace the original serine and threonine residues. To maintain the polarity of the amino acids in the position Y in the sequence Gly-X-Y, the hydroxylation of protocollagen proline has developed. The present mammalian α1-chain is the most stable.

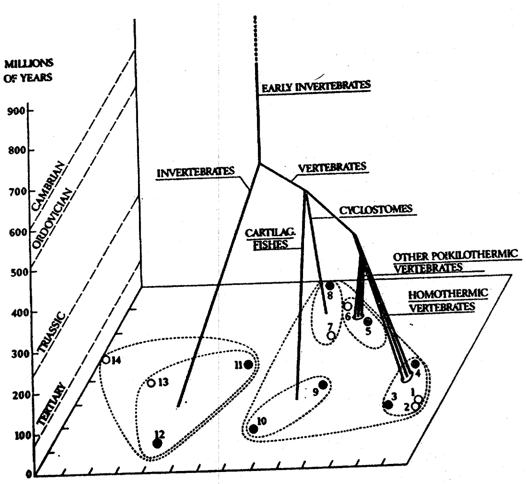

Fig. 1. Scheme on the divergent evolution of the collagen chains. (*6*). Key to numbering: 1 man, 2 monkey, 3 pig, 4 chick, 5 frog, 6 flounder, 7 lamprey, 8 hagfish, 9 dogfish, 10 rayfish, 11 squid, 12 sea mussel, 13 sea anemone, 14 earthworm.

The relationships of collagens can be demonstrated on the basis of amino acid composition (Table I). The larger the expected distance to the common ancestor, the larger the difference in the amino acid composition. Collagens can be grouped to a tentative scheme (Fig. 2) which includes also membranous collagens, secreted fibrous proteins and elastin (*61*). Resilin and silk fibroin (*73*) resemble the collagens of lower vertebrates.

It is gratifying to note that Dr. E. Miller from the Institute of Dental Health, Bethesda, Md., has been able to isolate and characterize a collagen from chicken cartilage in which all the three α-chains are similar as we found in the skin collagen from cartilagineous fishes. Details on the homology of collagen chains will be clarified by future sequence work.

Thus far the studies have been concerned mainly with the structure of tropocollagen, although Dr. J. Pikkarainen gives in his thesis (*8*) data on the superstructure to show that there has been evolution in the intermolecular bonding also (Table II). That fraction of cold-insoluble collagen,

TABLE I

Total variations in the amino acid composition of collagens from various sources

Elastin, resilin and silk fibroin are presented for comparison. The method of calculation is explained in ref. 7. The maximum of the value is 2000.

Species and tissue	Man	Pig	Chick	Frog	Flounder	Rayfish	Hagfish	Lamprey	Lens capsule	Squid	Sea mussel	Earthworm	Sea anemone	Elastin	Resilin	Silk fibroin
Man, umbilical cord	—															
Pig, skin	43	—														
Chick, skin	76	71	—													
Frog, skin	166	165	142	—												
Flounder, skin ..	200	195	170	62	—											
Rayfish, skin	196	205	214	180	182	—										
Hagfish, skin	240	241	226	104	72	170	—									
Lamprey, skin ..	166	165	138	54	60	166	96	—								
Cattle, ant. lens capsule	251	257	270	344	383	317	406	344	—							
Squid, body wall .	186	207	208	150	182	136	178	148	286	—						
Sea mussel, byssus	247	246	273	234	282	163	276	250	276	151	—					
Earthworm, body wall	278	275	292	238	271	199	268	255	338	214	180	—				
Sea anemone, body wall	276	269	282	259	290	181	297	266	277	137	132	213	—			
Elastin	602	597	584	594	624	598	630	620	620	624	589	620	620	—		
Resilin	414	425	458	314	316	332	274	350	511	348	316	354	391	658	—	
Silk fibroin	450	409	392	366	390	338	388	376	517	348	301	292	333	680	356	—

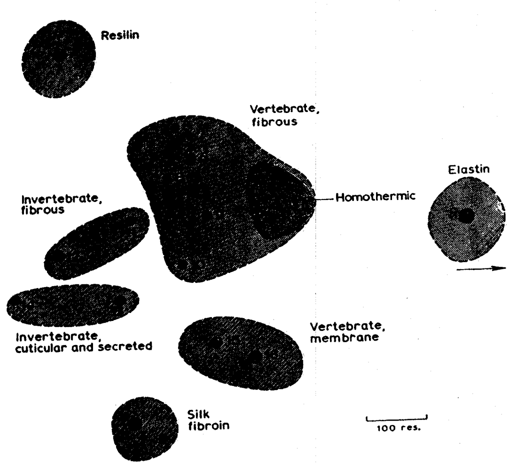

Fig. 2. Mutual relations of collagens from various species and tissues (*61*). Resilin, elastin and fibroin are included for comparison. Key to numbering: 1 man, 2 monkey, 3 pig, 4 chick, 5 flounder, 6 frog, 7 hagfish, 8 lamprey, 9 dogfish, 10 rayfish, 11 squid, 12 sea anemone, 13 sea mussel (byssus), 14 earthworm, 15 glomerular basement membrane of dog (*72*), 16 anterior lens capsule from cattle.

TABLE II

Development of the resistance to solvents and of the thermal stability of collagens from various sources (*8*)

T_S thermal shrinking, T_D thermal denaturation temperature

Species	Solubility*			Thermal stability	
	+40°	+65-90°	Residue	T_D°C	T_S°C
Cow	0.5	10.9	88.6	—	70—73
Calf	2.7	76.0	21.3	37.4	68—71
Guinea pig, adult ...	9.2	74.9	15.9	37.1	—
growing .	4.2	91.2	4.6	37.3	—
Chick	6.0	72.5	21.5	40.7	64—67
Snake	2.7	32.3	65.0	—	—
Frog	16.9	73.7	9.4	25.3	58—61
Burbot ...	69.3	24.1	6.6	19.8	—
Pike	78.4	17.7	3.9	26.0	—
Hagfish ..	55.9	26.9	17.2	15.7	59—62
Lamprey ..	89.9	7.5	2.6	21.0	65—68

* in % of cold-insoluble collagen into 0.01 *M* acetate buffer, pH 4.8.

which dissolves at +40° (cf. p. 18) and is not firmly cross-linked, is larger in animals with low thermal stability of collagen. In such cases a preliminary thermal shrinkage is observed at +30—55° (*8*). The evolution of the cross-linking would be a worthwhile topic of future research. More variability will be encountered on the supermolecular levels than in tropocollagen. On carbohydrates the knowledge is fragmentary as yet (*60*), but it is under intense investigation elsewhere (*73, 74*).

The expectations on the evolutionary work must be set for a long range, but that does not mean that it would have no economic or practical applications. The sustained work would give clues (*i*) on the essential features for collagens in various structures (fibres, sheets, membranes) and (*ii*) on the eventual utilization of gelatins as additional components in such structures, for example, to make them compatible with living tissues. Properties of such semiartificial materials could be varied through the proportions of the different components. Collagenous materials from now neglected products of Nature (*e.g.*, of fishes and invertebrates) might have unexpected but useful properties. The byssus-collagen of sea mussel, with its record-high thermal stability, is a good example of the natural modifications of collagen.

Basement membrane collagen

The common origin of membrane and fibrous collagens is evident from the SLS-patterns (Fig. 4) although clear differences exist (*62*). On the amino acid compositions the differences between mammalian fibrous and basement membrane collagens are rather large (Table I). The expected similarity with the cuticular or secreted invertebrate collagens is not very apparent. Fig. 2 shows the "best fit" of membranous collagens among other related proteins (*61*).

Composition and preparation of collagen fractions

Collagen components. The collagen samples can be preserved, *e.g.,* for transport in ethanol at +5° (*13*). The separation of the α- and β-components (*14*) by gel electrophoresis was applied also to preparative scale (*15*). Combined with gel filtration it is a very useful method for the separation of various degradation products of collagen (*30*; Fig. 8 below).

The temperature-dependent denaturation and renaturation of the different components of collagen have been demonstrated and T_D estimated in very illustrative way (Fig. 3) by Dr. T. Hollmén (*16, 17*) with starch-gel electrophoresis in a temperature gradient.

The preparation of various collagen fractions from granulation tissue has been worked out as also the separation and purification of the polymeric fractions. The apparently insoluble fraction was broken

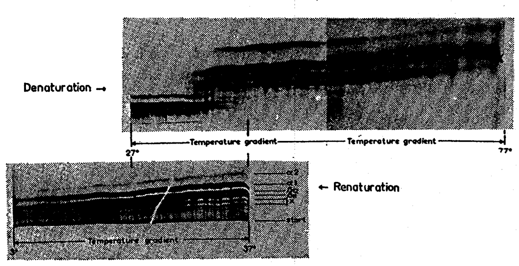

Fig. 3. Illustration of the denaturation and renaturation of collagen components in starch-gel electrophoresis at a perpendicular temperature gradient (*16, 17*).

down to α- and β-components during further preparation (*18*). The various collagen fractions, separated on their solubility, have a greatly variable component composition (*19*). Only the acetic acid-soluble fraction contains polymeric units. The α- and β-components differ at their salting-out (*20*). At pH 3.6 both components precipitated (except some α1-component) but at pH 4.4 the α1- and α2-components were partly left in the supernatant as also the β_{12}. These observations induce caution against the indiscriminate use of the component patterns in the characterization of collagen preparations.

Basement membrane of the lens capsule. The anterior lens capsule of cattle was dissected, treated with ultrasonics and extracted to remove soluble proteins. Collagen was solubilized with pepsin (1:200 in 1 % acetic acid—0.01 *N*-hydrochloric acid) at +4°. The gel electrophoretic pattern contains two strong bands, perhaps corresponding to the α-chains but not migrating exactly at the same speed as the α-chains from fibrous collagen. The bands show a conspicuously greyish tint at staining with Amido black. For the first time the whole molecule of membrane collagen could be reconstituted to SLS-particles. The electron microscopy (Fig. 4) shows a charge profile which resembles that of fibrous collagen but with obvious differences (*62*). The thermal denaturation temperature T_D was 34.6° and the thermal shrinking temperature T_S 52.5—54.5°C.

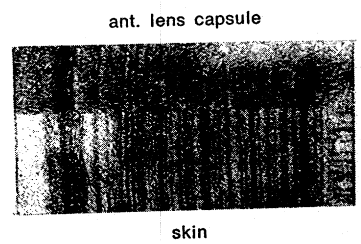

Fig. 4. Electron microscopic patterns of SLS-collagens of basement membrane from anterior lens capsule of cattle and of calf skin (*62*).

Gelatins. The technical gelatin preparations are very complex and the conventional ion-exchange methods yield very complicated fractionation patterns (*21*). The early work on the Amberlite CG-50-fractionated gelatins was continued in better controlled conditions (*22*). The resulting elution pattern depends, among other things, on the temperature employed for the gelatinization of insoluble collagen. The "pH 5.5-eluted" peak appears first after treatment at +120°, when the gradient-peak dominates. In extracts obtained at +65° and +90° the NaOH-eluted peaks were maximal. Recognizable α-components were released at +65° only.

The amino acid compositions of the different Amberlite CG-50-fractions resemble each other but certain differences should be noted (*22*). As expected, the "pH 5.5-eluted" fraction is most acidic. The NaOH-eluted fractions contain relatively much imino acids, especially non-hydroxylated proline (in the position next after glycine), but remarkably little lysine and no hydroxylysine (Table III). This fact was not noted in the paper, but may indicate that lysine is bound in the cross-links. These fractions should be investigated further applying the new methods to demonstrate lysine-based cross-links (*75*). In any case, those fragments which are eluted first with NaOH differ from the average collagen by the amino acid composition.

TABLE III

Contents of certain amino acids in gelatin fractions obtained by Amberlite CG-50-column chromatography (22)

The figures express residues per 1000.

Amino acid	Eluted at pH 5.5	Eluted by gradient buffer	Eluted by NaOH
Hypro	109	106	108
Pro	113	115	132
Gly	350	349	359
Hylys	2	3	0
Lys	28	24	15
Acidic residues	128	112	120
Basic residues .	69	78	51
Hypro/Pro ...	0.97	0.91	0.82
Imino residues	222	220	240
OH-groups ...	162	165	164

Determination of prolyl-hydroxyproline. When collagen was analyzed for its carbohydrate components by gas-liquid chromatography, an unexpected peak (Fig. 5) emerged which could not be identified with

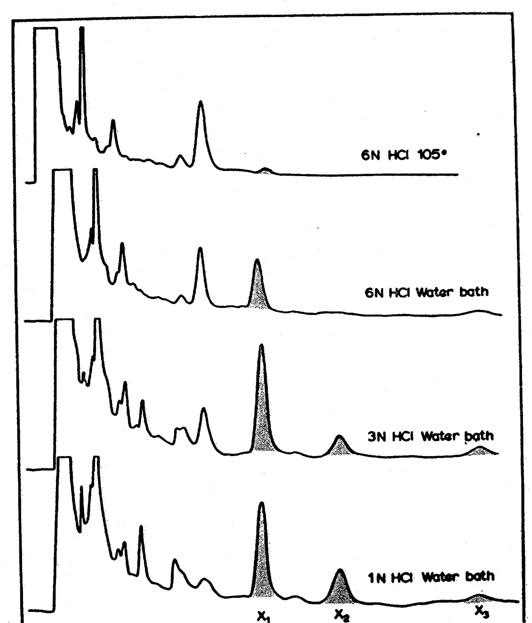

Fig. 5. Gas-liquid-chromatographic patterns of trimethylsilylated partial hydrolyzates of collagen. The shaded peak X_1 is the TMS-derivative of prolyl-hydroxyproline (*63*).

any sugar (*23, 63*). Incorporation experiments *in vivo* indicated that it contained the label from proline. From the reactivity with silylating agents and from the behaviour in ion-exchange columns it was inferred that the material could be converted to a form which did not carry free amino or carboxyl group but contained an OH-group. The unknown compound was thought to be the diketopiperazine of Pro-Hypro which was confirmed by the comparison with the synthetic substance with infrared and mass spectroscopy. Experiments with gradual hydrolysis of gelatin at various conditions showed that Pro-Hypro is totally liberated in 3—4 h at +100° in *N*-hydrochloric acid but itself is hydrolyzed very slowly (about 10 % in 10 h). With the use of internal standard it is possible to determine the peptide Pro-Hypro in any sequence. This procedure may be applied also as an analytical tool to follow the hydroxylation of protocollagen proline and in the estimation of the urinary excretion of Pro-Hypro.

Degradation with pepsin

The pepsin-digest of soluble rat-tail-tendon collagen gives a characteristic starch-gel electrophoretic pattern (*1*), if the treatment is done above +30° (*24, 25*). The $\alpha 2$-chain is more susceptible (*26*). The electrophoretic pattern is remarkably constant (*27*) after the treatment for several hours indicating that in collagen helix there are certain pepsin-sensitive points but that the majority of the bonds are relatively stable.

Combining various purification methods it has been possible to separate several fragments in pure form (*28—30*), renaturate five fragments to SLS-form and locate four of them in the tropocollagen macromolecule (*30, 31, 64*). The fifth (Fig. 10D) is the shortest fragment of collagen which has been ever renatured (about 200 Å) but it could not be located (Fig. 6—10, *64*).

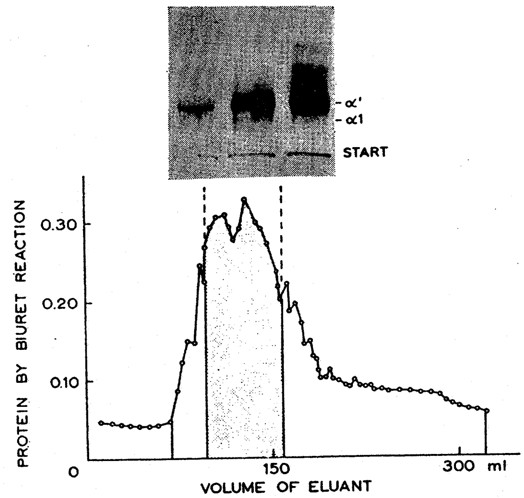

Fig. 6. Preparation of the fragment α' by rechromatography of the first Sephadex-G-200-fraction (*30*). The corresponding gel-electrophoretic patterns are presented for comparison.

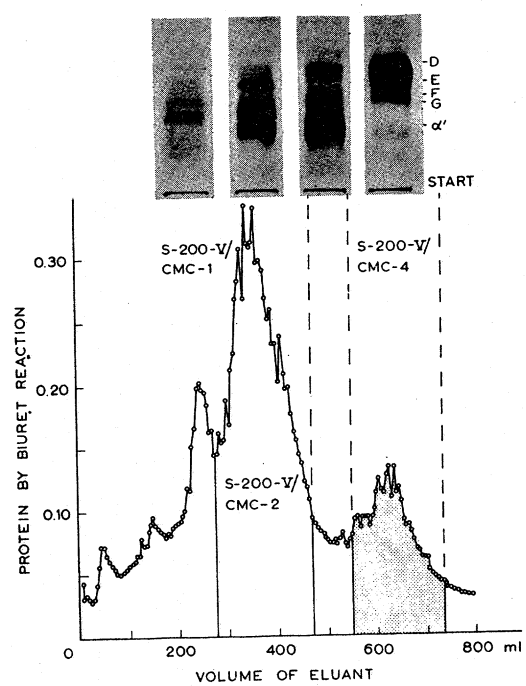

Fig. 7. Refractionation (with CMC-column) of the material obtained in the peak V from Sephadex-G-200-column (*30*).

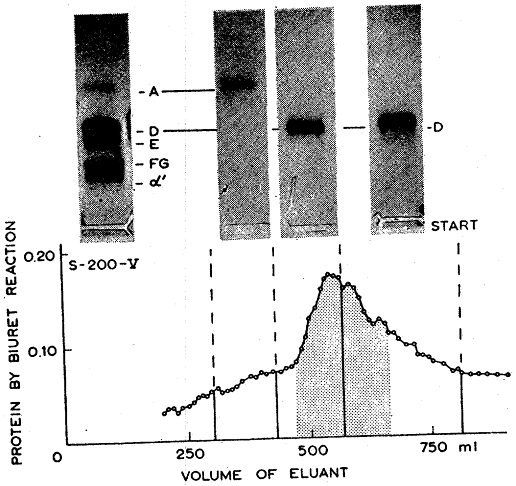

Fig. 8. Final purification of fragment D. The material from the shaded area in the pattern illustrated in Fig. 7 is refractionated by preparative electrophoresis (*15, 30*). Fragment A also is electrophoretically homogenous, but it can be divided into two subfractions by CMC-column.

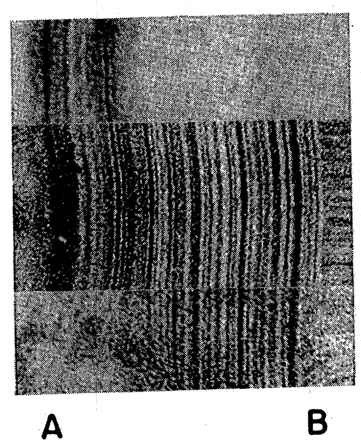

Fig. 9. Location of the fragments D (top) and α' (below) in the tropocollagen molecule (*31*).

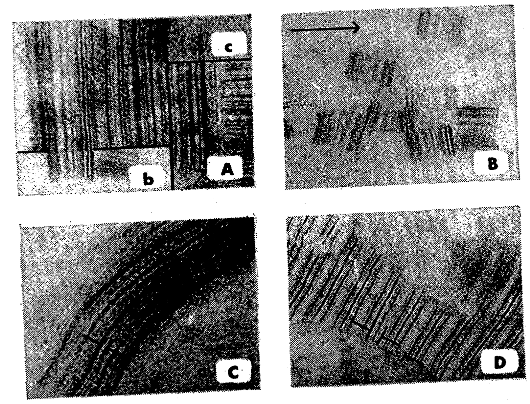

Fig. 10. Location of smaller pepsin-liberated fragments in the tropocollagen molecule (*64*). In the part A, fragments b (< B) and c (< C) are located beside SLS-tropocollagen. The length of the arrow 1000 Å.

In the course of this work we separated by phosphocellulose chromatography peptides which do not belong to the helical backbone of collagen but rather to the acidic structural proteins (Table IV). This procedure affords a novel approach to these widespread components of connective tissue (*76, 77*) which may be important in the future developments (see also next paragraph). These pepsin-liberated fragments resemble by amino acid composition the non-collagenous proteins reported in the literature (*78—80*). There is some similarity also with the telopeptide fraction, but discrepancies exist in regard to threonine, valine, phenylalanine and tyrosine.

For the sequence work there are now more exact chemical methods available for degradation. However, the enzymatic degradation will be useful in the studies on the supermolecular, especially densely cross-linked structures, perhaps in combination with gradual thermal degradation.

Associated non-collagenous proteins

Dr. A. Rajamäki has isolated and purified (Fig. 11, 12) from granulation tissue a group of heat-resistant glycoproteins, which are electrophoretically homogenous at wide pH-range and have a composition resembling the acidic structural glycoproteins by amino acid composition (Table IV, *32, 65, 76—80*). No hydroxyproline or hydroxylysine was detected, but abundantly tyrosine and phenylalanine, as also various hexoses and hexosamines. The sedi-

TABLE IV

Amino acid composition of certain non-collagenous peptide fragments from rat-tail tendon and experimental granuloma (64, 65)

The figures for S-200-VII-subfractions (*30*) are obtained by first estimating the contribution of collagen on the basis of hydroxyproline value and the remainder adjusted as residues per 1000. PC subfractions obtained by phosphocellulose column chromatography.

Amino acid	Sephadex-G-200-VII-subfractions of pepsin-digested rat-tail tendon			Non-collagenous protein from granuloma (*65*)	"NGF-1-P" of intervertebral disc (*78*)	Urea-extracted protein of rat-skin (*76*)	"PPL-3" of nasal cartilage (*79*)	Pepsin-liberated telopeptide (*80*)
	PC-(1+2)	PC-4	PC-5					
Hypro ..	0	0	0	0	0	0	0	0
Asp	109	98	126	99	103	93	68	95
Thr	35	27	35	62	58	51	60	0
Ser	77	62	59	57	65	55	127	49
Glu	134	169	128	123	107	156	132	141
Pro	76	108	66	66	67	41	89	82
Gly	143	101	108	82	100	83	126	144
Ala	66	39	62	71	68	89	83	33
½Cyss ..	0	1	8	—	—	14	tr	0
Val	53	36	42	71	59	55	63	0
Met	13	3	3	17	25*	29	3	0
Ileu	36	27	27	48	38	55	40	0
Leu	86	84	69	92	86	98	84	128
Tyr	63	72	54	31	33	29	11	144
Phe	104	56	30	36	41	34	34	115
Hylys ..	0	0	0	—	9	0	0	0
Orn	0	0	0	—	2	0	0	0
Lys	5	62	79	62	42	58	29	10
His	0	8	25	20	19	16	19	20
Arg	0	47	79	61	35	31	33	39
Trp	—	—	—			13	—	—
Unknown.	++	+	±		++	—	—	—

* includes cystine; tr traces

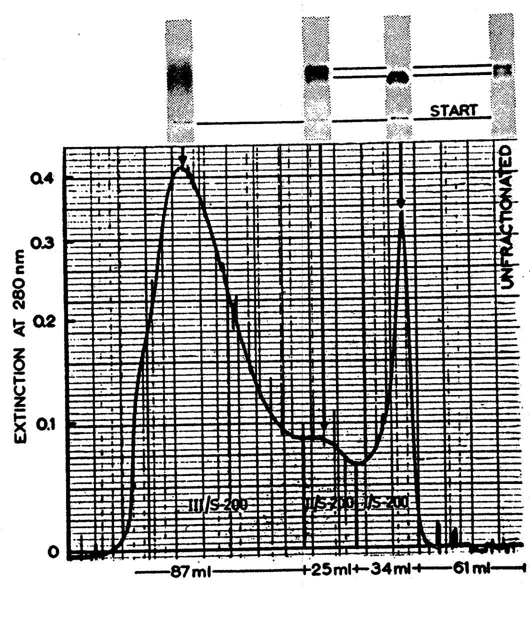

Fig. 11. Fractionation of the non-collagenous material from granulation tissue by DEAE-column chromatography. The two fractions can be separated in preparative scale (*65*).

mentation coefficient is 3.1S and the isoelectric point about pH 4.2. Three similar collagenase-resistant fractions were obtained also from skin, one of them obviously elastin. The skin of an old rat contains more of this fraction, and it is related to the two fractions of insoluble collagen (cf. p. 18), which dissolve at different temperatures.

The role of proteins of this kind in the formation of stable collagen is not yet understood. They seem to enhance the aggregation of collagen *in vitro* (unpublished results).

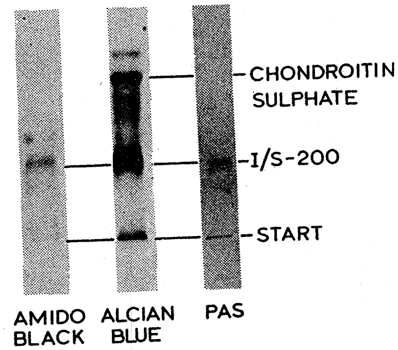

Fig. 12. Electrophoretic patterns on cellulose acetate membrane of the material obtained in DEAE-cellulose column chromatography referred to in Fig. 11. The contaminations with Alcian blue-positive material is revealed (*65*).

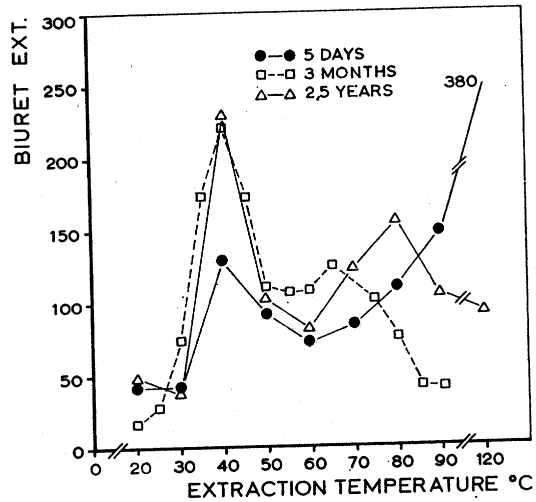

Fig. 13. Extractability of insoluble collagen from the skins of rats of various ages at various temperatures (*34*).

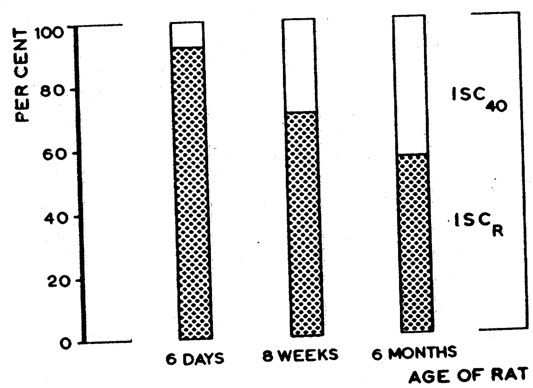

Fig. 14. Proportions of the two components of insoluble collagen in skins of rats of various ages (*34*). ISC$_R$ collagen extractable above +80°.

Effect of age on the structure of collagen

When the gelatinization of insoluble skin collagen was made at +100°, the material from young guinea pig yielded at the Amberlite CG-50 fractionation more "pH 5.5-eluted" material than the corresponding preparation from adult rats (*33*). The main fraction was in both cases gradient-eluted. The NaOH-eluted fractions were larger when the collagenous material originated from old animals. Combinations of these methods (extractions at various temperatures, Amberlite CG-50 and phosphocellulose chromatography) can be utilized in order to isolate those fragments of collagen which have been influenced by age-dependent processes, for example, on the cross-linking.

The "cold-insoluble" collagen can be divided into two fractions (*34*) (*i*) not or weakly cross-linked, gelatinizable at +40° and (*ii*) firmly cross-linked which dissolves first at about +80° as breakdown products. The fraction extracted above +70° contains less of the reactive aldehyde groups. The first fraction, with the weaker cross-links, is proportionally increased with the age. The insoluble collagen of the younger animals contains more associated hexosamine than that of the old animals (*35*).

When insoluble collagen from chick embryos, pretreated with labelled proline, was gelatinized and then fractionated with Amberlite CG-50 column, the "pH 5.5-eluted" fraction was the most radioactive (*36*), indicating that it arises from the surface regions of the fibres which have been most degraded during the longest contact with the extracting solution. The metabolic heterogenity of insoluble collagen should be stressed.

Synthesis of collagen

Molecular biology. The report on the first grant period (*1*) contained data on the three metabolic phases in collagen-synthetizing granuloma tissue (*37*), which experience has been reviewed elsewhere (*38*). The remarkable point is that the periods of cellular proliferation and collagen synthesis can be separated at various experimental applications (*66*).

The synthesis of collagen is disturbed by some antibiotics which affect protein synthesis (*39*) as also by substances which inhibit the synthesis of nucleic acids (*40*), although it is not known whether fibroblasts differ in the sensitivity from other cells. Dr. J. Ahonen has been able to prepare from the fibroblasts fractions of RNA which could be the "messenger-RNA" of collagen (*41*). The nucleotide compositions of those fractions are changed during the period of collagen synthesis (*42*). We have not been able to demonstrate a synthesis of collagen by homogenate *in vitro* (*43*).

As an incidental finding it may be added that the turnover of $\alpha 2$-chain is faster than that of $\alpha 1$ (*35*), which is also more stable metabolically (Fig. 15) (cf. p. 10).

Amino acids, lactate. The synthesis of collagen depends on the extracellular concentration of proline and glutamic acid (*44*) (Fig. 16). In our system the other amino acids are not limiting. The formation of proline from glutamic acid coincides with the period of collagen synthesis in the slices. Data have been collected but not yet analyzed on the intracellular concentra-

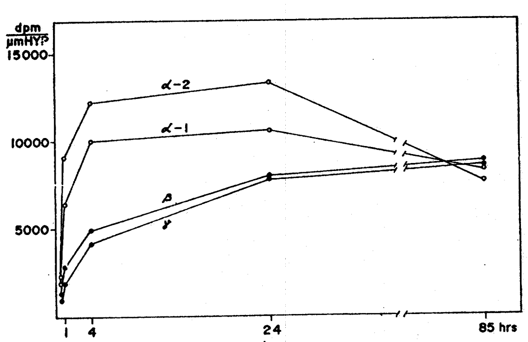

Fig. 15. Specific radioactivities of $\alpha 1$- and $\alpha 2$-chains as function of time (*35*).

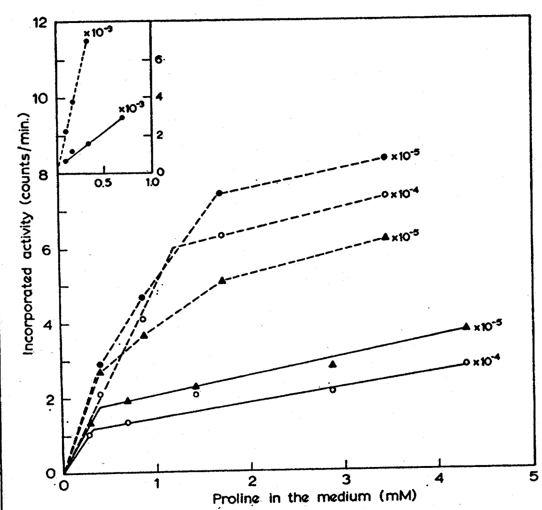

Fig. 16. Requirement of extracellular proline for the protein synthesis in the granulation tissue (*44*). ○ Hydroxyproline, ● gelatinized protein, ▲ non-collagenous protein, ———, ... different methods for the preparation of the samples.

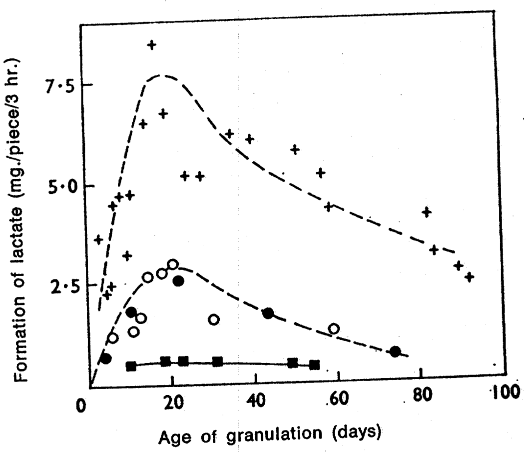

Fig. 17. Production of lactate by slices of experimental granulation tissue (*37*). The maximum coincides with the maximal capacity to synthetise collagen. ■—■—■ no glucose in the medium.

tion of amino acids in the fibroblasts at the different developmental phases.

Glucose is necessary for the synthesis of collagen and the maximal production of lactic acid coincides with the capacity of collagen synthesis *in vitro* (*37*, Fig. 17). Obviously the redox-balance is important for the synthesis of collagen. The transport of proline to cells is shown to require in micro-organisms the presence of lactic acid. The conversions of α-ketoglutaric acid first to glutamic acid and then to proline are dependent on the redox-state, as also the hydroxylation of protocollagen proline. Indeed, the effect of lactic acid on the synthesis of collagen can be demonstrated directly (*44*, Table V). Similar results have been obtained also by Comstock & Udenfriend (*81*) with fibroblast culture.

TABLE V

Effect of added lactate on the incorporation of glutamate to collagen in granuloma slices (*44*)
The activities are expressed in counts/min/100 mg of slices. The %-effect of lactate is given in the parentheses.

Precursor	Lactate m*M*	Collagen (hydroxyproline)	Non-collagenous protein
Glutamic acid, 0.72 m*M*	0	870	84600
	10	1220 (+40 %)	139100 (+65 %)
Glutamic acid, 1.42 m*M*	0	1170	114200
	10	1680 (+43 %)	157000 (+37 %)
Glutamic acid, 2.87 m*M*	0	1290	—
	10	1620 (+25 %)	—
Proline, 2.87 m*M*	0	22890	288800
	10	29580 (+29 %)	316200 (+10 %)

Collagen fibres

Aggregation. An extensive series of aggregation experiments has been made in various conditions according to Gross & Kirk (*82*) by following the turbidity of precipitating tropocollagen as a function of time (*67a, b*).

The aggregation is strongly pH-dependent, modestly accelerated by added gelatin. All alcohols retard the aggregation even in molar concentrations of 1:1000 in water. The effect is increased with the chain length, but is independent on the concentration of collagen. Organic acids accelerate the aggregation at molar concentrations of 1:1000—400 but retard it at higher concentrations. All the amino acids accelerated the aggregation with the exception of proline and hydroxyproline (Table VI). The effect of serine was the same as that of alanine.

As known previously (*82, 83*), urea and arginine retarded the aggregation, and benzoylarginine and the enzyme substrates benzoylarginylamide and benzoylarginine ethyl ester even more. These experiments were made to test the hypothesis that collagen may form intermolecular complexes analogously to the binding of serine enzymes with their substrates. The results support but do not prove the hypothesis.

TABLE VI

Effect of hydroxylic compounds on the aggregation of tropocollagen (*67a, b*)

The relative time interval to the formation of the half-maximal turbidity is given (at no addition $t_{\frac{1}{2}} = 1.0$). Cf. Fig. 18.

Addition	Molar ratio of additive to water		
	1:1000	2.5:1000	5:1000
Methanol	1.0	1.1	1.2
Ethanol	1.2	1.8	3.3
Propanol-1	1.5	3.2	>4.0
Butanol-1	2.1	>4.0	>4.0
Pentanol-1	2.2	>3.8	—
Ethandiol-1,2 ...	1.2	1.5	2.7
Propandiol-1,3 ..	1.6	3.6	>4.0
Butandiol-1,4 ...	1.9	>4.0	>4.0
Glycerol	1.4	2.5	>4.0
Sorbitol	1.6	6.1	—
Serine	0.7	0.4	—
Threonine	0.8	0.6	—
Hydroxyproline .	1.1	1.2	1.3

Sodium and potassium ions had little effect, but other cations tested (Li, Ca, Mg, Sr, Ba, Co) retarded the aggregation very strongly (Table VII). Mono-, di- and triphosphates all accelerated the aggregation but, surprisingly, ATP retarded it (Fig. *18*).

The material will be treated with the help of a physicochemist and crystallo-

TABLE VII

Effect of cations on the aggregation of tropocollagen (*67a, b*)

The experimental arrangement is the same as referred to in Table VI and Fig. 18.

Addition	Molar ratio of additive to water		
	1:1000	2.5:1000	5:100
LiCl	1.4	2.7	>7.3
KCl	1.0	0.8	0.6
NaCl	1.1	1.0	0.8
$CaCl_2$	2.4	>7.3	—
$MgCl_2$	3.4	>8.0	—
$SrCl_2$	7.1	>7.3	—
$BaCl_2$	>7.3	—	—
$CoCl_3$	0.6	2.4	>7.3

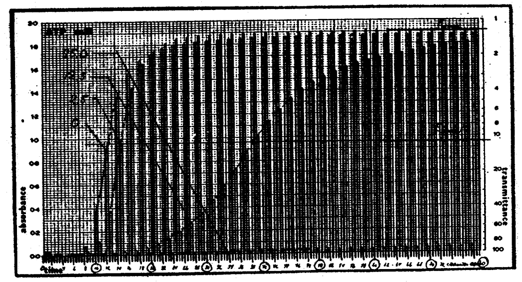

Fig. 18. Retarding effect of added ATP on the aggregation of tropocollagen (*67*). Note the increase of $t_{\frac{1}{2}}$ = time interval to the half-maximal turbidity at increasing concentrations of ATP.

grapher. As the model we will use, first at least, Wood's formulas on the nucleation and growth of collagen (*84 a—c*).

Maturation to insoluble form. Most of the work has been done by Dr. E. Heikkinen (*35, 45 a, b*). The maturation is more effective in young animals (Table VIII, *35*).

TABLE VIII

Effect of age on the incorporation of proline into collagen fractions of rat skin (*35*)

NSC neutral salt soluble collagen, ASC acetic acid-soluble collagen, ISC insoluble collagen.

Age	Hydroxyproline (cpm/g fresh skin)	NSC/ASC	NSC/ISC
6 days	17 560	25.2	11.8
8 weeks	11 440	59.3	79.0
6 months	8 860	257.0	565.0

Calcium ion is necessary for the formation of insoluble collagen (Fig. 19). Other metal cations could be listed in the following order of decreasing efficiency: Co > Cu > Au > Ni > Fe^{3+} > Cr > Ca > Fe^{2+} > Al > Bi > Pb > Sn > Sr > Ba,Mg,Mn > Li,Na,K (*34, 45 b*). The carbonyl-fixing reagents as semicarbazide inhibit the formation of insoluble collagen *in vitro* (*34*).

During the assay of the "maturation" (*45 a, 68*) thin strips of skin from 2 month-

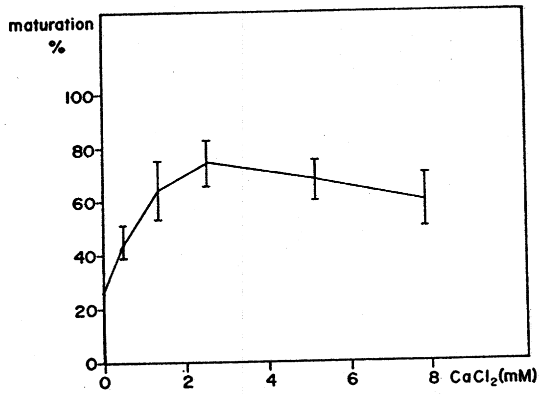

Fig. 19. Requirement of Ca^{2+} for the formation of insoluble collagen *in vitro* (*34*).

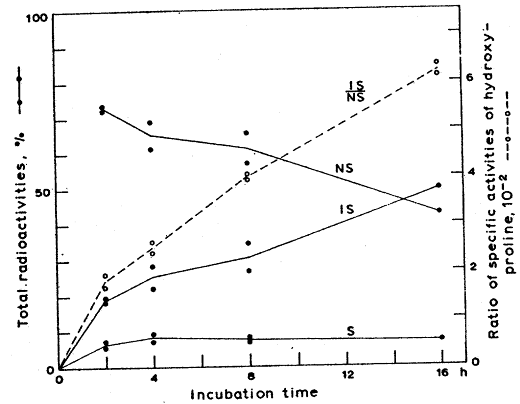

Fig. 20. Demonstration of the formation of insoluble collagen (IS) from neutral salt-soluble collagen (NS) (*68*).

old rats pretreated with labelled proline are incubated with a skin-homogenate from 4—6 day-old rat. The strips are rinsed and their collagen fractionated to neutral salt-soluble (NS) and insoluble (IS) fractions. The ratio of specific radioactivities IS/NS is used as the index of "maturation" (Fig. 20). Thus far the method is tedious, but it may have some advantage in the studies on the significance of tissue components *in situ,* especially in regard to the non-collagenous glycoproteins which are associated with collagen in insoluble structures. The enzymic reaction itself depends presumably on the deamination of lysines to the corresponding aldehyde (*85 a, b*), but the limiting factors in the tissues are not known.

APPENDIX*

REGULATION OF FIBROBLAST ACTIVITIES

Experiments mainly in vivo

Production of fibroblastic tissue. The various methods to study the fibroblasts in solid tissue have been especially elaborated (*38, 66*). The formation of collagen-producing colony of fibroblasts, *i.e.* experimental granulation tissue, can be provoked by steel-wire net to avoid artificial temporal effects. Even then there is a lag period before the synthesis of collagen as in the sponge-induced granuloma and the peak of the lactic acid formation coincides with the peak of the collagen synthesis. One-dimensional growth of the fibroblasts can be observed with an implanted sponge-filled tube, where the zones of connective tissue penetration can be noticed. The growth in such a tube is limited but can be stimulated by a cut through the recently developed tissue (Fig. 21). The mechanism of the stimulation to proliferate is unknown. The tube granuloma can be applied to testing the effects of substances which would be washed away from the implanted sponge.

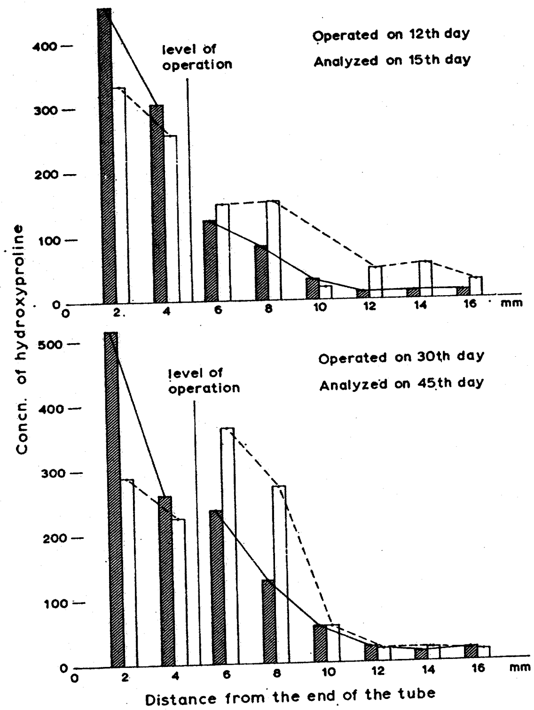

Fig. 21. Effect of cutting the granulation tissue at the indicated level during its development into a tube. The shaded columns and solid lines indicate the non-operated control, the open columns and dashed lines the operated granulomas (*66*).

Age. The age of the animals, from which the fibroblasts originate, influences their metabolism (*46, 69*). In general, fibroblasts of a younger generation pass all the functional phases (proliferation, collagen synthesis, involution) more rapidly and perform the metabolic activities with higher intensity per cell. The rate of the cell proliferation is not generation-dependent but rather the translation of the genetic information to the synthesis of enzymes as also of structural proteins as collagen.

Hormonal effects. The hormones of adrenal cortex have a especially strong effect on the formation of collagen *in vivo*. We are convinced that the synthesis of

* Not a part of the project UR-E8-(60)-17.

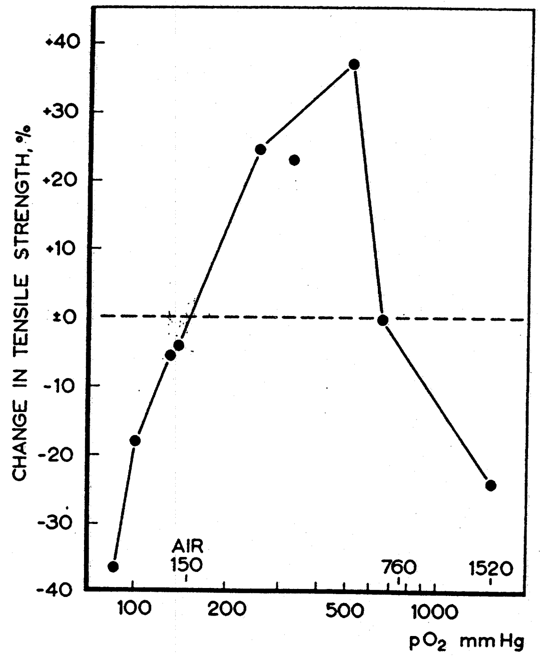

Fig. 22. Effect of oxygen tension in the breathing gas on the formation of the tensile strength in the healing skin wound (*52*).

collagen itself is not affected but rather the development of the fibroblasts (*1, 47*). The effects of hypophysectomy and growth hormone have been studied in detail by Dr. M. Valavaara with growing animals (*48, 49*). The positive significance of growth hormone and also of thyroxine on the synthesis of collagen are obvious, especially on the amounts of the soluble forms.

Oxygen supply. Dr. J. Niinikoski has studied particularly the effect of the composition of the breathing gas on the formation of collagen (*50—54*). There is an optimal supply of oxygen (Fig. 22) and above that appear toxic manifestations (*51, 54*). We believe that the mechanism of the beneficial effect is related, partly at least, with the increased formation of RNA in the cells (*52, 53*) (Fig. 23), accompanied by an accelerated differentiation of the protein synthesis in the fibroblasts. Studies on the mechanism of the toxicity will be pursued further both in the lung and in the granulation tissue.

Irradiation. The local irradiation of collagen-producing granulation tissue results in a decrease of the soluble forms of collagen but, in contrast, the insoluble fractions increase (*55*). There are temporary changes also in the DNA-fractions: there appears a more soluble form, presumably a breakdown product.

Glycosaminoglycans, calcification. The study on the content and metabolism of glycosaminoglycans in relation to the formation of collagen fibres in the connective tissue has been initiated (*56*). Especially keratan sulphate and acid glycoprotein fractions seem to be attached to the insoluble collagen. An interesting point is the short biological half-life (*57*). The composition of the fracture callus and the subsequent bone has been studied and the calcification followed (*58*). The supply of oxygen, either hypoxia or hyperoxia, affects the calcifica-

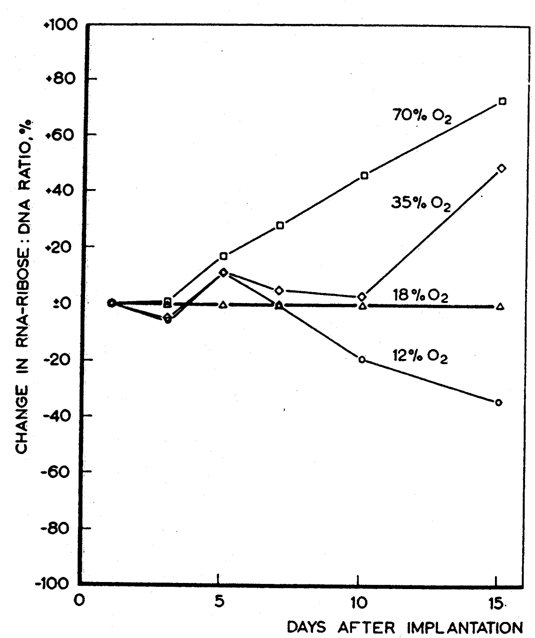

Fig. 23. Effect of oxygen content in the breathing gas on the formation of RNA per cell (*52*).

tion more than the synthesis of mucopolysaccharides or collagen in the cartilage (*70*).

General effects of mesenchymal reaction. A mesenchymal reaction, also in the form of an experimental granuloma, is reflected in the whole body. During the collagen synthesis in the granuloma the urinary excretion of methylated purines increases (*59a*). It is under investigation whether the methylated purines originate from granuloma itself or from other tissues, for example, liver. The effects of cell proliferation (chronic inflammations, pregnancy or malignant growth) on plasma protein fractions may be due to similar indirect mechanisms. The development of the experimental granulation tissue induces an elevated level of protein-bound hydroxyproline in plasma (*59b*) but little change is seen in the urinary excretion of hydroxyproline.

Experiments mainly in vitro

On the method. We are convinced on certain advantages of slices of granulation tissue over fibroblast culture as a research tool. The contacts of the cells to other cells and to surrounding macromolecules (collagen, glycoproteins and acid mucopolysaccharides) are kept intact and their regulatory effects maintained. The variations of experimental granuloma are suitable for studies (*i*) on the kinetics of a fibroblast population, (*ii*) on the production of collagen and other components of connective tissue, (*iii*) on the relation of cell proliferation to the synthetic functions and gene expression, (*iv*) on the differentiation and phase sequences in the synthesis of various proteins, (*v*) on the reparation and wound healing, (*vi*) on the effects of physiological manipulations (*e.g.*, supply of nutrients and of oxygen) and of physical factors (*e.g.*, irradiation), and finally (*vii*) on the actions of poisons and drugs.

Serotonin and indole compounds. A series of connective tissue-active drugs has been studied with the incubated slices of granulation tissue on the incorporation of proline to collagen and other proteins. Several welknown anti-inflammatory substances (acetyl salicylate, myochrisin, aminophenazon) are quite inactive, but phenylbutazon and indomethacin instead very effective in suppressing these cell functions in concentrations of $10^{-3}M$—$10^{-4}M$. The point of interest is that also other substances with indole nucleus, *e.g.*, serotonin, affect the fibroblasts (*86*) and this is confirmed with granulation tissue slices (Fig. 24) (*71*). We know from the literature that even indole itself inhibits the synthesis of acid mucopolysaccharides in cartilage. At low concentrations ($10^{-5}M$—$10^{-6}M$) both serotonin and indomethacin stimulate the synthesis of collagen (but not of other proteins) in granulation tissue slices.

The point of further interest is that neuraminic acid is claimed to be a receptor of serotonin. The interesting chain of events from serotonin (indole compounds) through neuraminic acid to collagen synthesis is thus far unknown in detail.

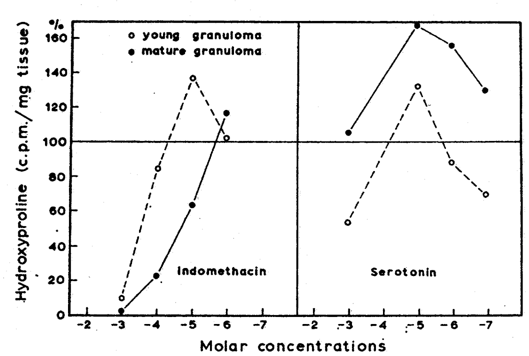

Fig. 24. Effect of indomethacin and serotonin in various concentrations on the incorporation of proline into collagen in granuloma slices. The respective control level = 100 (*71*).

TABLE IX

Effect of pretreatment of mature granuloma slices with various enzymes on the subsequent incorporation of proline to collagen and other proteins (71)

The figures express % of respective controls.

Addition	A. Collagen (hydroxy-proline)	B. Non-collagenous protein	Ratio A/B
Hyaluronidase ..	87	78	1.1
" ..	73	112	0.7
Papain	54	42	1.3
"	97	70	1.4
"	100	66	1.5
Trypsin	42	24	1.8
Neuraminidase ($n = 9$), several preparations of various origin ..	64.0±4.6**	107.4±7.1**	0.62*

* difference from 1.00 significant, $P < 0.001$; ** difference between A and B (non-independent pairs) significant ($P < 0.005$).

Pretreatment with neuraminidase. Because the charges of acid mucopolysaccharides seem important for the regulation of the functions of the fibroblasts, the acidic groups on the cell surface due to neuraminic acid were modified (*71*). After experiments with several neuraminidase preparations it can be concluded, that neuraminidase-treated fibroblasts synthatise much less collagen than the controls but that the synthesis of other proteins is not affected (Table IX). Thus the neuraminic acid-containing moiety in the cell wall is on the receptor which regulates the gene expression and affects specifically the synthesis of a single protein. This is in agreement with the suggested role of glycolipids in the transformations of virus-treated fibroblasts.

Ouabain, electrolyte concentrations. Ouabain, which inhibits the K,Na-activated adenosine-triphosphatase in the cell wall, disturbs the synthesis of collagen especially (*44*) (Table X). A perhaps related effect can be demonstrated by the variations in the concentrations of sodium and potassium which both are necessary and their total concentration is also significant. The remarkable point is that the synthesis of collagen is affected much more than the synthesis of the intracellular proteins (Table XI). As a conclusion it may be stated that the synthesis of collagen depends on the metabolism on the cell wall in somewhat specific way.

TABLE X

Effect of ouabain on the incorporation of proline to collagen in mature granuloma slices (44)

The figures express % of respective control.

Age of granuloma days	Concn. M	A. Collagen (hydroxy-proline)	B. Non-collagenous protein	Ratio A/B
7	10^{-4}	65	73	0.89
7	10^{-3}	15	33	0.45
20	10^{-4}	57	93	0.61
21	10^{-4}	64	95	0.68
20	10^{-3}	41	—	—

Lipoproteins and immunoglobulins. When granulation tissue slices were incubated in Krebs-Ringer-phosphate medium in the presence of Cohn's fractions I—III or of very-low-density lipoproteins a sti-

TABLE XI

Effect of ambient electrolyte concentrations on the incorporation of proline to collagen and other proteins in mature granuloma slices (44)

The figures express % of respective control (Na 125, K 5 mM).

Na^+ mM	K^+ mM	A. Collagen (hydroxy-proline)	B. Non-collagenous protein	Ratio A/B
		20-day granulomas		
0	125	33	74	0.45
120	0	32	107	0.30
120	5	100	100	1.00
100	100	70	93	0.76
50	50	300	300	1.00
		7-day granulomas		
0	125	19	30	0.63
125	0	13	52	0.25
120	5	100	100	1.00

mulation in the incorporation of proline to collagen hydroxyproline was observed ($+35.5$ %, $P < 0.01$). Future research will show whether this finding is relevant to the formation of scleroses and fibroses or to the development of cirrhosis in fatty liver where the synthesis of collagen is increased analogously.

When various preparations of γ-globulins were added to the incubation medium in the concentrations of 1 %, the synthesis of collagen was suppressed in proportion to other proteins (-24.6 %, $P < 0.01$). Other globulins were without effect. The whole pattern remains to be elaborated but is of obvious interest and importance.

Silica, acid mucopolysaccharides, serum albumin. The dust lung is caused almost specifically by silicium particles, but no effects are produced by quartz *in vitro*. However, silica gel seems to stimulate the functions of the fibroblasts in slices ($+49.7$ %, $P < 0.001$).

The acid mucopolysaccharides are closely related to the cell wall. At least heparin stimulates the fibroblasts to synthetise collagen ($+39.3$ %, $P < 0.025$). This conclusion is confirmed by the inhibition of the synthetic functions at the addition of protamine which binds acid mucopolysaccharides. The eventual analogous effect of hyaluronate and chondroitin sulphates cannot be assessed with this method because the medium becomes too viscous. Their effects will be studied with tube granuloma (p. 23).

If serum albumin is added in the medium up to 3 % there seems to be a stimulation of collagen synthesis. Future work will show whether this depends on the charges of the protein or on the colloid osmotic pressure.

Comments

The preliminary evidence presented in the Appendix suggests that the cell wall is important in the specific regulation of the synthesis of collagen and other proteins. This fundamental biological question can be generalized to the regulation of cell proliferation *vs.* synthesis of extracellular proteins. With fibroblast and collagen the problem can be approached with concrete experiments. The ultimate aim should be the development of a model of the cell wall to explain its receptor functions in the regulation of the gene expression and other cellular activities.

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I wish to acknowledge my spiritual debt to my former teacher, Professor A. I. Virtanen, Helsinki, and to the "grand old man" of collagen research, Professor K. H. Gustavson, Stockholm, for their inspiring support.

As the Appendix this report contains material of more biological nature which has been obtained by the continuous support from the Sigrid Jusélius Foundation and it is impossible to separate the two projects sharply. The confidence of this generous foundation has been felt as an obligation to keep in mind the ultimate purpose of our work: to reveal the structure and function of living organisms to the benefit of Man in health and disease.

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REFERENCES

Reports of this and previous project

1. *Research work on the structure and metabolism of collagen.* Final report on the project UR-E8-(60)-3, Turku, 1965 (33 pages).
2. *First Annual Report,* August 31, 1966 (10 pages and 8 enclosures).
3. *Second Annual Report,* August 31, 1967 (15 pages and 9 enclosures).
4. *Third Annual Report,* August 31, 1968 (16 pages and 5 enclosures).
5. *Fourth Annual Report,* August 31, 1969 (17 pages and 12 enclosures).

Printed publications on the results of this project

6. Kulonen, E. & Pikkarainen, J. (1970). Comparative studies on the chemistry and chain structure of collagen. In *The Chemistry and Molecular Biology of the Intercellular Matrix* (E. A. Balazs, ed.) *1,* 81—97. London: Academic Press.
7. Pikkarainen, J. & Kulonen, E. (1969). Comparative chemistry of collagen. *Nature, 223,* 839—841.
8. Pikkarainen, J. (1968). The molecular structures of vertebrate skin collagens. Thesis. *Acta physiol. Scand.,* suppl. *309,* 1—72.
9. Pikkarainen, J., Rantanen, J., Vastamäki, M., Lampiaho, K., Kari, A. & Kulonen, E. (1968). On collagens of invertebrates with special references to *Mytilus edulis. European J. Biochem., 4,* 555—560.
10. Pikkarainen, J. & Kulonen, E. (1967). On the evolution and development of collagen. *Scand. J. clin. Lab. Investigation,* suppl. *95,* 40.
11. Pikkarainen, J. & Kulonen, E. (1968). On the evolution and development of collagen. *Seventh Internat. Congress of Biochemistry,* Tokyo, Abstract J-225.
12. Penttinen, R., Kari, A. & Kulonen, E. (1967). Starch-gel electrophoresis in the characterization of products of collagen breakdown. *Acta chem. Scand., 21,* 827.
13. Pikkarainen, J. & Kulonen, E. (1966). Preservation of native collagen. *Suomen Kemistilehti* (*J. Finn. chem. Ass.*), *B 39,* 156—157.
14. Kulonen, E., Hollmén, T., Näntö, V. & Pikkarainen, J. (1965). Experience on the starch gel electrophoresis of heat-denatured collagens. In *Structure and Function of Connective and Skeletal Tissue* (S. Fitton—Jackson *et al.,* eds.), p. 216—221. London: Butterworths.
15. Hollmén, T. & Kulonen, E. (1966). Separation of the components in collagen by preparative starch-gel electrophoresis. *Analyt. Biochem., 14,* 455—466.
16. Hollmén, T. & Kulonen, E. (1966). Combination of temperature gradient with gel electrophoresis and its applications to analysis of collagen-gelatin transition. *J.Chromatog., 21,* 454—459.
17. Kulonen, E. & Hollmén, T. (1966). An illustrative method for demonstration of denaturation-renaturation of subunits of collagen.. *Fed. Proc., 25,* 1030—1031.
18. Heikkinen, E., Valavaara, M., Penttinen, R. & Kulonen, E. (1967). The preparation of various components of collagen. *Ann. Med. exp. Fenn., 45,* 259—263.
19. Heikkinen, E. & Kulonen, E. (1966). Collagen components in the consecutive extracts of rat skin. *Acta physiol. Scand., 68,* 231—233.
20. Heikkinen, E. & Kulonen, E. (1966). Precipitation of the collagen components by salts. *Acta chem. Scand., 20,* 914—915.
21. Pikkarainen, J. & Kulonen, E. (1965). The fractionation of various gelatin preparations with ion-exchange resins. *Suomen Kemistilehti* (*J. Finn. chem. Ass.*), *B 38,* 226—230.
22. Pikkarainen, J. & Kulonen, E. (1968). Composition of fractions of denatured collagen resolved on Amberlite CG-50. *Acta chem. Scand., 22,* 1267—1270.
23. Lampiaho, K., Nikkari, T., Pikkarainen, J. & Kulonen, E. (1970). Gas-chromatographic analysis of prolyl-hydroxyproline in collagen hydrolyzates. *Scand. J. clin. Lab. Investigation,* suppl. *113,* 36.
24. Lampiaho, K., Kari, A. & Kulonen, E. (1968). Fractionation of pepsin-digested denatured collagen. *Scand. J. clin. Lab. Investigation,* suppl. *101,* 3.
25. Penttinen, R., Kari, A. & Kulonen, E. (1966). Starch-gel electrophoretic pattern of pepsin-treated collagen. *Acta chem. Scand., 20,* 1304—1308.
26. Penttinen, R., Kari, A. & Kulonen, E. (1968). The susceptibility of the alpha-1 and alpha-2 components of collagen to pepsin. *Ann. med. exp. Fenn., 46,* 185—187.
27. Lampiaho, K., Kari, A., Niinikoski, J. & Kulonen, E. (1966). Time course of the action of pepsin on insoluble and soluble collagens. *Acta chem. Scand., 20,* 1446—1448.
28. Pikkarainen, J., Hollmén, T., Lampiaho, K., Kari, A. & Kulonen, E. (1968). Characterization of the two large fragments from pep-

sin-digested denatured collagen. *Scand. J. clin. Lab. Investigation*, suppl. *101*, 3.

29. LAMPIAHO, K., KARI, A., HOLLMÉN, T., PIKKARAINEN, J. & KULONEN, E. (1967). Preparative work on pepsin-digested denatured collagen. *Biochem. J.*, *104*, 21P.

30. LAMPIAHO, K., NIINIKOSKI, J., KARI, A. & KULONEN, E. (1968). Fractionation of pepsin-digested, denatured collagen. *Biochim. biophys. Acta*, *160*, 77—89.

31. PIKKARAINEN, J., HOLLMÉN, T., LAMPIAHO, K., KARI, A., & KULONEN, E. (1968). Characterization of the large fragments from pepsin-digested, denatured collagen. *Biochim. biophys. Acta*, *160*, 90—95.

32. RAJAMÄKI, A. & KULONEN, E. (1969). Isolation of a glycoprotein from collagenase-digested insoluble sponge-granulomas. *Scand. J. clin. Lab. Investigation*, suppl. *108*, 22.

33. KULONEN, E. & PIKKARAINEN, J. (1968). The reflection of age, tissue and solubility on the Amberlite CG-50 fractions of denatured collagens. *Acta physiol. Scand.*, *74*, 10—15.

34. HEIKKINEN, E. (1968). Transformations of rat skin collagen with special reference to the ageing process. Thesis. *Acta physiol. Scand.*, suppl. *317*, 1—69.

35. HEIKKINEN, E. & KULONEN, E. (1968). Effect of age on the maturation of rat-skin collagen. *Biochim. biophys. Acta*, *160*, 464—466.

36. LAMPIAHO, K., JUVA, K. & KULONEN, E. (1969). Incorporation of amino acids into fractions of insoluble collagen. *Ann. Med. exp. Fenn.*, *43*, 254—256.

37. LAMPIAHO, K. & KULONEN, E. (1967). Metabolic phases in the development of granulation tissue. *Biochem. J.*, *105*, 333—341.

38. KULONEN, E. (1970). Studies on experimental granuloma. In *The Chemistry and Molecular Biology of the Intercellular Matrix* (E. A. Balazs, ed.) *1*, 1811—1820. London: Academic Press.

39. AINE, E. & KULONEN, E. (1969). Effect of some antibiotics in wound healing. *Scand. J. clin. Lab. Investigation*, suppl. *108*, 18.

40. KARI, A. & KULONEN, E. (1969). The effect of cyclophosphamide and methylaminopterin on wound healing and experimental granuloma. *Scand. J. clin. Lab. Investigation*, suppl. *108*, 21.

41. AHONEN, J. & KULONEN, E. (1967). Messenger RNA in experimental granulomas. *Scand. J. clin. Lab. Investigation*, suppl. *95*, 40.

42. AHONEN, J. (1968). Nucleic acids in experimental granuloma. Thesis. *Acta physiol. Scand.*, suppl. *315*, 1—73.

43. KUMENTO, A., KARI, A., HOLLMÉN, T. & KULONEN, E. (1966). On the intracellular synthesis of collagen. *Ann. Med. exp. Fenn.*, *44*, 58—62.

44. AALTO, M., LAMPIAHO, K. & KULONEN, E. (1970). Amino acid metabolism of granuloma slices. *Scand. J. clin. Lab. Investigation*, suppl. *113*, 35.

45a. HEIKKINEN, E. & KULONEN, E. (1967). The maturation of rat skin collagen *in vitro*. *Scand. J. clin. Lab. Investigation*, suppl. *95*, 40.

45b. HEIKKINEN, E., IVASKA, K. & KULONEN, E. (1968). Effect of metal cations on the stability of collagenous structure *in vitro*. *Scand. J. clin. Lab. Investigation*, suppl. *101*, 5.

46. HEIKKINEN, E., AALTO, M. & KULONEN, E. (1970). Age factor in the formation of experimental granulation tissue. *Scand. J. clin. Lab. Investigation*, suppl. *113*, 35.

47. MIKKONEN, L., LAMPIAHO, K. & KULONEN, E. (1966). Effect of thyroid hormones, somatotrophin, insulin and corticosteroids on synthesis of collagen in granulation tissue both *in vivo* and *in vitro*. *Acta Endocrinologica*, *51*, 23—31.

48. VALAVAARA, M. & KULONEN, E. (1969). The pituitary control of granulation tissue. *Scand. J. clin. Lab. Investigation*, suppl. *108*, 23.

49. VALAVAARA, M., HEIKKINEN, E. & KULONEN, E. (1968). Effect of hypophysectomy on the incorporation of proline in the collagen fractions. *Experientia*, *24*, 779—780.

50. KULONEN, E., NIINIKOSKI, J. & PENTTINEN, R. (1967). Effect of the supply of oxygen on the tensile strength of healing skin wound and granulation tissue. *Acta physiol. Scand.*, *70*, 112—115.

51. NIINIKOSKI, J. & KULONEN, E. (1968). Hyperbaric oxygenation in wound healing and experimental granuloma. *Scand. J. clin. Lab. Investigation*, suppl. *101*, 111.

52. NIINIKOSKI, J. (1969). Effect of oxygen supply on wound healing and formation of experimental granulation tissue. Thesis. *Acta physiol. Scand.*, suppl. *334*, 1—72.

53. NIINIKOSKI, J. & KULONEN, E. (1970). Reparation at increased oxygen supply. *Experientia*, *26*, 247—248.

54. NIINIKOSKI, J., NIKKARI, T. & KULONEN, E. (1971). Composition of endobronchial saline extracts of rats during exposure to oxygen. *Aerospace Med.*, in press.

55. RANTANEN, J. & KULONEN, E. (1969). Effect of x-ray irradiation of granulation tissue. *Scand. J. clin. Lab. Investigation*, suppl. *108*, 22.

56. LEHTONEN, A. (1968). The mucopolysaccharides in ageing experimental granulation tissue. Thesis. *Acta physiol. Scand.*, suppl. *310*, 1—78.

57. IVASKA, K., HEIKKINEN, E., LEHTONEN, A. & KULONEN, E. (1969). Metabolism of acid glycosaminoglycans in granulation tissue. *Acta chem. Scand.*, *23*, 3615—3617.

58. PENTTINEN, R. & KULONEN, E. (1969). The chemical composition of fracture callus in rat. *Scand. J. clin. Lab. Investigation*, suppl. *108*, 22.

59a. VASTAMÄKI, M. V., AHONEN, J. & KULONEN, E. (1968). Effect of experimental granuloma on the urinary excretion of methylated purines in rat. *Scand. J. clin. Lab. Investigation*, suppl. *101*, 9.

59b. KUMENTO, A. & KULONEN, E. (1967). Reflection of collagen synthesis in experimental granulation tissue of plasma protein-bound hydroxyproline. *Scand. J. clin. Lab. Investigation*, *19*, 298—300.

Manuscripts

60. RÖNNEMAA, T., PIKKARAINEN, J. & KULONEN, E. Carbohydrates associated with collagen preparations from various species. *Suomen Kemistilehti B* (*J. Finn. Chem. Ass*), in press.

61. PIKKARAINEN, J. & KULONEN, E. Evolutionary relations of various collagens, resilin, fibroin and elastin. A manuscript.

62. PIKKARAINEN, J. & KULONEN, E. (1971). Structural studies on the basement membrane collagen. Abstract. *Scand. J. clin. Lab. Investigation,* in press.

63. LAMPIAHO, K., NIKKARI, T., PIKKARAINEN, J. & KULONEN, E. Gas-chromatographic analysis of prolyl-hydroxyproline in partial hydrolyzates of collagen and gelatin. A manuscript.

64. PIKKARAINEN, J., LAMPIAHO, K. & KULONEN, E. Fractionation of pepsin-digested, denatured collagen. III. Characterization of small and associated non-helical fragments. A manuscript.

65. RAJAMÄKI, A. & KULONEN, E. Purification of a glycoprotein from collagenase-digested sponge-granulomas. A manuscript.

66. RANTANEN, J., LAMPIAHO, K. & KULONEN, E. Applications of experimental granulation tissue. In preparation.

67a. PIKKARAINEN, J. & KULONEN, E. Factors affecting the aggregation of tropocollagen. In preparation.

67b. PIKKARAINEN, J. & KULONEN, E. The effect of low-molecular substances on the fibre formation of collagen. *Z. klin. Chem.,* in press.

68. IVASKA, K., HEIKKINEN, E. & KULONEN, E. Method for studies on the formation of insoluble collagenous structures. In preparation.

69. HEIKKINEN, E., AALTO, M., VIHERSAARI, T. & KULONEN, E. Age factor in the formation and metabolism of experimental granulation tissue. *J. Gerontology,* in press.

70. PENTTINEN, R., NIINIKOSKI, J. & KULONEN, E. Hyperbaric oxygenation and fracture healing. A biochemical study with rats *in vivo. Acta chir. Scand,* in press.

71. AALTO, M. & KULONEN, E. Effect of neuraminidase, serotonin and indomethacin on the synthesis of collagen and other proteins in slices of experimental granuloma. Abstract. *Scand. J. clin. Lab. Investigation,* in press.

Other relevant publications

72. KEFALIDES, N. A. & DENDUCHIS, B. (1969). Structural components of epithelial and endothelial basement membranes. *Biochemistry, 8,* 4613—4621.

73. RUDALL, K. M. (1968). Comparative biology and biochemistry of collagen. In *Treatise of Collagen* (B. S. GOULD, ed.), 2A, p. 83—137. London: Academic Press.

74. GRANT, M. E., FREEMAN, I. L., SCHOFIELD, J. D. & JACKSON, D. S. (1969). Variations in the carbohydrate content of human and bovine polymeric collagens from various tissue. *Biochim. biophys. Acta, 177,* 682—685.

75. BAILEY, A. J., PEACH, C. M. & FOWLER, L. J. (1970). Chemistry of the collagen cross-links. Isolation and characterization of two intermediate intermolecular cross-links in collagen. *Biochem. J., 117,* 819—831.

76. TIMPL, R., WOLFF, I. & WEISER, M. (1969). Acidic structural proteins of connective tissue. I. Solubilization and preliminary chemical characterization. *Biochim. biophys. Acta, 194,* 112—120.

77. ROSS, R. & BORNSTEIN, P. (1969). The elastic fiber. I. The separation and partial characterization of its macromolecular components. *J. Cell Biol., 40,* 367—381.

78. STEVEN, F. S., JACKSON, D. S. & BROADY, K. (1968). Proteins of the human intervertebral disc. The association of collagen with a protein fraction having an unusual amino acid composition. *Biochim. biophys. Acta, 160,* 435—446.

79. SCHUBERT, M. & HAMERMAN, B. (1968). *Primer on Connective Tissue Biochemistry.* Philadelphia: Lea and Febiger.

80. DRAKE, M. P., DAVISON, P. F., BUMP, S. & SCHMITT, F. O. (1966). Action of proteolytic enzymes on tropocollagen and insoluble collagen. *Biochemistry, 5,* 301—312.

81. COMSTOCK, J. P. & UDENFRIEND, S. (1970). Effect of lactate on collagen proline hydroxylase activity in cultured L-929 fibroblasts. *Proc. Nat. Acad. Sci., 66,* 552—557.

82. GROSS, J. & KIRK, D. (1958). The heat precipitation of collagen from neutral salt solution: some rate-regulating factors. *J. biol. Chem., 233,* 355—360.

83. COOPER, A. (1970). Thermodynamic studies of the assembly *in vitro* of native collagen fibrils. *Biochem. J., 118,* 355—365.

84a. WOOD, G. C. & KEECH, M. K. (1960). The formation of fibrils from collagen solutions. 1. The effect of experimental conditions: Kinetic and electron-microscope studies. *Biochem. J., 75,* 588—598.

84b. WOOD, G. C. (1960). The formation of fibrils from collagen solutions. 2. A mechanism of collagen-fibril formation. *Biochem. J., 75,* 598—605.

84c. WOOD, G. C. (1960). The formation of fibrils from collagen solutions. 3. Effect of chondroitin sulphate and some other naturally occurring polyanions on the rate of formation. *Biochem. J., 75,* 605—612.

85a. SIEGEL, R. C., PAGE, R. C. & MARTIN, G. R. (1970). The relative activity of connective tissue lysyl oxidase and plasma amine oxidase on collagen and elastin substrates. *Biochim. biophys. Acta, 222,* 552—555.

85b. SIEGEL, R. C., PINNELL, S. R. & MARTIN, G. R. (1970). Cross-linking of collagen and elastin. Properties of lysyl oxidase. *Biochemistry, 9,* 4486—4492.

86. BOUCEK, R. J. & ALVAREZ, T. R. (1970). 5-Hydroxytryptamine: a cytospecific growth stimulator of cultured fibroblasts. *Science, 167,* 898—899.